WHOLE BLOOD ROBOTIC CHOLINESTERASE ASSAY FOR ORGANOPHOSPHATE EXPOSURE - TESTING SOLDIERS, FIRST RESPONDERS, AND CIVILIANS IN THE FIELD AND LABORATORY

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1. ABSTRACT

Exposure to organophosphate (OP) chemical warfare agents (CWAs), pesticides, anesthetics, drugs such as cocaine, and a variety of therapeutic drugs including donepezil or rivastigmine for Alzheimer's disease reduces red blood cell acetylcholinesterase (RBC-AChE) or serum butyrylcholinesterase (BChE) activity. The activity of RBC-AChE and BChE can be used as potential biomarkers of suppressed and/or heightened function in the central and peripheral nervous systems. For instance, the toxicity of pesticides is well documented in humans. Therefore, blood cholinesterase (ChE) activity can be exploited as a tool for confirming exposure to these agents and possible treatments. Yet it is the OP CWAs that are some of the most potent and irreversible inhibitors that can produce excessive accumulation of acetylcholine, and a cholinergic crisis in man leading to paralysis and ultimately death.

Current assays for measurement of erythrocyte-bound acetylcholinesterase (RBC-AChE) and serum butyrylcholinesterase (BChE, pseudocholinesterase) require several labor-intensive processing steps, suffer from wide statistical variation, and inter-laboratory comparison is often difficult. Techniques currently used include the Ellman and microEllman microtiter method, radiometric, amperometric, and Δ (delta) pH (modified Michel protocol) used by the U.S. Army's Cholinesterase Reference Laboratories (CRL). Such methods determine only the serum BChE or RBC-AChE, and require the use of specific ChE inhibitors or sample processing such as centrifugation.

In contrast, the Walter Reed Army Institute of Research Whole Blood (WRAIR WB, U.S. Patent #6,746,850, June 8, 2004) cholinesterase assay (a) rapidly determines the activity of *both* AChE and BChE in unprocessed (uncentrifuged) whole blood, (b) uses a minimally invasive blood sampling technique (e.g., blood from a finger prick), and (c) is semi-automated for high-throughput using the Biomek 2000 robotic system (see figure 1). The method measures the activity of whole blood in the presence of three AChE and BChE substrates from which the individual AChE and BChE activities are calculated.

To date, the WRAIR whole blood assay has been used to measure AChE and BChE in human blood that has been inhibited by selective (e.g. huperzine A) and non-selective (carbamate) inhibitors (e.g. pyridostigmine bromide, PB). Both studies supported Federal Drug Administration (FDA) clinical trials. We found that volunteers given PB (30 mg single dose) exhibited RBC-AChE maximal inhibition of about 27% after 2.5 h, with recovery of activity to almost 100% after 24 h. After *ex vivo* addition of the OP nerve agent soman (GD) to PB-pretreated human blood, and subsequent PB and GD removal using a small spin column, all the protected (by PB pretreatment) AChE activity was recovered within 6 h. Thus, this assay provided validated support for PB pretreatment as protection against organophosphate chemical warfare agents. In the second FDA clinical trial, the WRAIR WB ChE assay was used to determine the RBC-AChE and serum BChE profile of healthy elderly volunteers receiving huperzine A. We observed about 53% RBC-AChE inhibition and no BChE inhibition at the end of an increasing dose regimen (final dose 200 µg bid in the fourth week). Huperzine A was well tolerated by these patients and warrants study as a future prophylaxis for OP poisoning.

Due to the documented use of OPs by terrorists and in warfare around the globe, Federal, State, and local authorities need a reliable, fast, inexpensive, and standard method for confirming such an assault in order to initiate

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appropriate containment, decontamination, and treatment measures. Thus, the WRAIR whole blood cholinesterase assay, with its high-throughput robotic capability, is ideal for prescreening military personnel for ChE activities that might preclude their deployment to areas of potential CWA exposure. This assay fulfills the requirement for rapid and reliable monitoring of OP exposure in military and civilian populations.

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2. INTRODUCTION

Cholinesterases are highly polymorphic carboxylesterases that display broad substrate specificity and are involved in the termination of neurotransmission in cholinergic synapses and neuromuscular junctions of the central nervous system (CNS). ChEs are classified as acetylcholinesterase and butyrylcholinesterase according to their substrate specificity and sensitivity to selected inhibitors (Silver, 1974). The concentration of AChE and BChE in blood is potentially a stable biomarker of suppressed and/or heightened central and peripheral nervous system activity. Exposure to nerve agents, OPs, pesticides, anesthetics, terrorists' chemical agents, cocaine, and some neurodegenerative disease states and their treatments selectively reduces AChE or BChE activity. In humans, the toxicity of pesticides is well documented (Lockridge and Masson, 2000). Therefore, blood cholinesterase activity can be exploited as a tool for confirming exposure to the agents and possible therapeutics (Taylor, 1990).

The current tests used to determine cholinesterase levels in blood, however, are not U.S. Food and Drug Administration approved and have significant drawbacks including the lack of standardization, long turn-around times, and difficulty in comparing results between alternate laboratories utilizing different ChE assays that report values in different or non-standard units. In part, this is because clinical determination of ChE levels in blood typically utilize three different techniques: Ellman, Michel (Δ pH), and pH stat, and normally determine either RBC-AChE or serum BChE concentrations, but not *both*. (St. Omer and Rottinghaus, 1992) In addition, radioactive assays for AChE activity, although very sensitive, require special handling and disposal and are not suitable for field use.

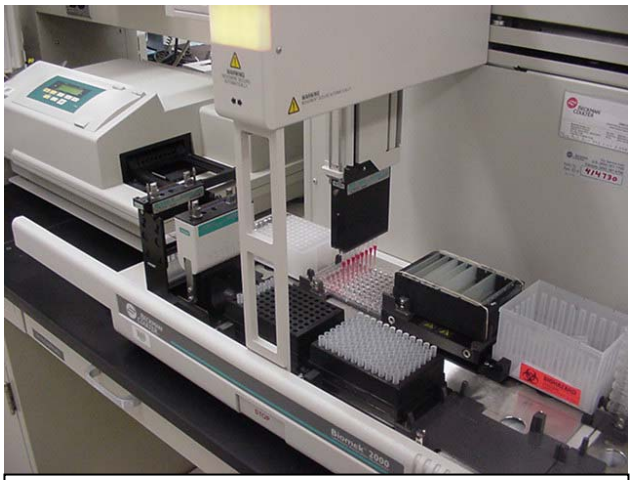

Figure 1. WRAIR whole blood robotic assay, consisting of the Biomek 2000, UV microplate reader, and computer processing (see section 3.1 and 3.2).

Therapeutic drugs have a wide effect on cholinesterases (Jann et al, 2002) and minor inhibition is observed with a variety of anesthetics. High inhibition is observed (purposely) with pyridostigmine bromide, its therapeutic advantage being to increase the muscle strength in myasthenia gravis patients by inhibiting acetylcholinesterase that results in an accumulation of acetylcholine at cholinergic synapses (Juhn, 1993). Yet it is the organophosphate chemical warfare agents that are some of the most potent and irreversible inhibitors that can produce excessive accumulation of acetylcholine, and ultimately a cholinergic crisis in man leading to death (Taylor, 1990). Although the physiological state of an individual, drugs, pesticides, and chemical warfare agents affect cholinesterases, only for the latter two are routine cholinesterase measurements utilized.

Studies on the toxicology of nerve agents and on treatment of intoxications have predominantly focused on lethal and supralethal doses of the agent. However, the possible relationship between the so-called Gulf War Syndrome and accidental exposure to trace amounts of sarin and cyclohexyl sarin (Ember, 1996) has made clear that knowledge on the acute and delayed effects of low or trace exposure to nerve agents, insecticides and a variety of environmental chemicals is scarce (Gardner et al, 2003; Abou-Donia et al, 1996). Several military and terrorist scenarios can be envisioned in which low or trace exposures become significant.

Given the potential increase in urban terrorism that may include the use of chemical warfare organophosphate agents, Federal, State, and local authorities need a reliable, fast, inexpensive, and standard method for confirming such an assault in order to initiate appropriate containment, decontamination, and treatment measures. To this end, we have developed a new methodology - The Walter Reed Army Institute of Research Whole Blood (WRAIR WB) cholinesterase assay - that quickly and accurately determines the activities of AChE and BChE

simultaneously in unprocessed, whole blood (U.S. Patent #6,746,850, 2004; Feaster et al, 2000; Haigh et al, 2004). The WRAIR WB assay measures the activity of whole blood in the presence of three substrates for AChE and BChE, which provide redundancy and independent determination of both AChE and BChE activities. This is possible because: 1) each protein possesses a different affinity and sensitivity for each of its substrates and 2) a direct relationship exists between activity and enzyme concentration.

Currently, the WRAIR WB assay has been used to support two FDA clinical trials. The first study was to demonstrate that pyridostigmine bromide pretreatment protected RBC-AChE against the OP chemical warfare agent soman (GD). In the second study, huperzine A, a potential therapeutic for Alzheimer's disease (Jiang et al, 2003), was shown to specifically inhibit RBC-AChE without adverse effects in normal elderly volunteers. The methods and results described below support the conclusion that the WRAIR WB assay is ideal for rapid and reliable monitoring of OP exposure in military and civilian populations and also monitoring therapeutic regimens for neurological diseases e.g., myasthenia gravis or Alzheimer's, where ChE inhibitors are used.

3. METHODS

3.1 The WRAIR whole blood (WB) assay theory and design. Current clinical determination of cholinesterase levels includes the Michel, pH stat, Ellman, and microEllman methodologies (Silver, 1974). These methods require several processing steps, normally determine either the serum or RBC cholinesterase activities but not both, and suffer from statistical error. For instance, in order to determine the RBC AChE levels, the Michel method (Δ pH) requires centrifugation to pellet RBCs followed by washing to remove serum BChE from the pellet prior to analysis of the sample for AChE. Since the different assay techniques measure different parameters (e.g., Δ pH per hour for the Michel assay in comparison to absorbance change per minute for the Ellman assay), inter-lab comparison has not been established. In addition to the clinical methodologies, a field deployable unit is commercially available, the Test-Mate OP™ system (EQM Research Inc., Cincinnati, OH). Although this unit is designed for field use, a selective BChE inhibitor is required to measure AChE. Therefore, two blood samples are necessary, which results in longer processing times for complete AChE and BChE screening.

The WRAIR WB method does not rely on the addition of selective AChE or BChE inhibitors, uses a single minimally-invasive blood collection technique of a finger prick (equally acceptable would be a small blood sample from a tube of *uncentrifuged* blood kept at 4°C or frozen), is not labor intensive, and produces rapid results due to automation. Thus, we have circumvented the aforementioned problems and simultaneously determined the levels of both AChE and BChE. This is possible because blood contains two cholinesterases that possess different affinities for any given substrate, and a linear correlation exists between enzyme activity and concentration. Thus, if one determines the activity in any given blood sample with two different substrates, then it is feasible to calculate the precise concentrations of both proteins (i.e., two equations with two unknown variables) (Bieberich and Yu, 1999). Furthermore, monitoring the activity with three different substrates (Figure 2) provides three fold degenerate data (i.e., three sets of two equations with two unknown variables). In figure 2, the rates of substrate hydrolysis are represented by R_1, R_2, and R_3 and correspond to the turnover of substrate 1, substrate 2, and substrate 3, respectively. The [AChE] and [BChE] refer to the actual activities of AChE and BChE in the sample. Finally, the coefficients in each equation (*i.e.*, x_1, x_2, x_3, and y_1, y_2, y_3) represent sensitivity coefficients and are the contribution that AChE and BChE independently contribute to the overall rate of hydrolysis of each substrate (R_1, R_2, R_3). Simultaneously solving these three sets of degenerate equations provides three independent estimates for the concentrations of AChE and BChE. Therefore, determining the mean value and the standard deviation for these independently derived values provides the concentrations of each protein, AChE and BChE.

$$x_1[AChE] + y_1[BChE] = R_1 \quad \text{Substrate 1}$$
$$x_2[AChE] + y_2[BChE] = R_2 \quad \text{Substrate 2}$$
$$x_3[AChE] + y_3[BChE] = R_3 \quad \text{Substrate 3}$$

$$[AChE]_{1,2} = \frac{\begin{vmatrix} x_1 & R_1 \\ x_2 & R_2 \end{vmatrix}}{\begin{vmatrix} x_1 & y_1 \\ x_2 & y_2 \end{vmatrix}} \quad \text{and} \quad [\text{AChE}]_{1,3} = \frac{\begin{vmatrix} x_1 & R_1 \\ x_3 & R_3 \end{vmatrix}}{\begin{vmatrix} x_1 & y_1 \\ x_3 & y_3 \end{vmatrix}} \quad \text{and} \quad [\text{AChE}]_{2,3} = \frac{\begin{vmatrix} x_2 & R_2 \\ x_3 & R_3 \end{vmatrix}}{\begin{vmatrix} x_2 & y_2 \\ x_3 & y_3 \end{vmatrix}}$$

$$\therefore mean[AChE] = \frac{[AChE]_{1,2} + [AChE]_{1,3} + [AChE]_{2,3}}{3}$$

The [BChE] can be calculated in a similar manner.

Figure 2. Mathematical representation of the sensitivity coefficient method described in 3.1.

3.2 WRAIR WB Assay conditions. The final concentrations of the substrates for the 96-well microtiter plate in the WRAIR WB assay were 1 mM each of acetylthiocholine iodide (ATC), propionylthiocholine iodide (PTC), butyrylthiocholine iodide (BTC), and 0.2 mM 4,4' dithidiopyridine (DTP), the indicator for the hydrolyzed thiocholine (UV absorbance at 324 nm). To perform the ChE assays, human whole blood was collected from subjects and stored with heparin at 4°C or frozen at -80°C. Note that hemoglobin from lysed blood does not interfere with the assay at this wavelength, thus providing a greater signal to noise ratio (data not shown, Feaster, 2000). A

small aliquot of blood, typically 10 µL, which was diluted 20-fold in distilled water, was placed in the well to give a final volume of 300 µL using 50 mM sodium phosphate buffer, pH 8.0 containing the appropriate substrate and DTP, and performed in triplicate. Following a sixty second pre-read shaking to mix the contents thoroughly, a four-minute kinetic assay was performed (at 25°C) on each plate using a Molecular Devices SpectraMax Plus^{384} microtiter spectrophotometer (Sunnyvale, CA), interfaced to a Beckman-Coulter Biomek 2000 robotic station (Fullerton, CA), that performed all the plate and sample handling steps. Each well was read at twelve second intervals, interspersed with three second shaking. The data were subjected to linear least squares analysis, from which the activities of AChE and BChE (U/mL) were calculated using SoftMax v4.6 and an Excel spreadsheet (figure 3). The spreadsheet also documents samples and substrate batches for GLP record keeping.

3.3 Measurement of pyridostigmine bromide induced ChE inhibition: Aliquots of whole blood from twenty-four volunteers (nineteen given a single oral dose of pyridostigmine bromide (30 mg tablet) and five placebos) were withdrawn for the determination of RBC-AChE and serum BChE activity. A blood sample was taken before the PB dose (0 time or prescreen) and then at 2.5, 5, 8 and 24 h after dosing. Blood samples were frozen at –80°C, and thawed immediately prior to the WRAIR WB cholinesterase assay to limit decarbamylation (i.e., reversal of ChE inhibition by PB).

3.4 Measurement of huperzine A induced ChE inhibition (RBC-AChE): Aliquots of whole blood from twelve healthy elderly volunteers given an increasing dose regimen of huperzine A (50 µg twice daily for one week, then increasing doses of 100 µg, 150 µg, and finally 200 µg twice daily in Week 4) were withdrawn at various time intervals up to 24 h for RBC-AChE and serum BChE activity determination. Blood was sampled immediately prior to the first huperzine A dose and 24 h after the last (200 µg) dose. Three individuals served as controls and were given a placebo. As for PB-containing blood, samples were frozen (–80°C) and thawed immediately prior to the WRAIR WB cholinesterase assay.

3.5 Measurement of protection afforded to RBC-AChE by PB or huperzine A to *ex vivo* soman exposure: To evaluate the protection afforded to RBC-AChE by PB or huperzine A, blood samples stored at –80°C were thawed and then exposed to the irreversible OP soman (1 µM) for 10 min at room temperature; these experiments were conducted at USAMRICD, Aberdeen Proving Ground, MD). Free PB or huperzine A and GD were removed from the blood by using Biorad spin columns (6 cm) containing 300 mg of C_{18} (Waters, Milford, MA, #WAT010001). After adding 2.5 µL of saponin (50 mg/mL) and vortexing for 1 min, 180 µL of the lysed whole blood, was added to the column and centrifuged at 1,000 x g for 2 min. In addition to removing free ligands from the blood, almost 100% of the AChE/BChE activity from the original blood sample is recovered. Although these samples were not exposed to oximes, interfering compounds including oximes such as 2-PAM and HI-6 can also be removed (data not shown). Thus, after PB or huperzine A removal, we are able to monitor the time taken to achieve full return in activity (decarbamylation for PB or dissociation from the active site by huperzine A), and how much of the RBC-AChE is protected from GD exposure. The % recovery of ChE activity is calculated as follows: % inhibited (PB/huperzine A or placebo samples) = 100 x (ChE U/mL of samples at times post-dosing)/(ChE U/mL pre-dosing). The % recovery (PB/huperzine A or placebo samples exposed to GD) = 100 x (ChE U/mL of GD samples at times post-dosing)/(ChE U/mL pre-dosing).

3.6 HPLC Assays of PB and huperzine A. To correlate AChE-RBC inhibition with blood concentrations of PB or huperzine A, or to verify compliance of PB consumption (for example troops anticipating potential OP exposure), we have developed a sensitive HPLC technique to quantify PB (Garcia and Moorad-Doctor, 2004) and huperzine A in human blood (unpublished). The PB HPLC technique is based on solid phase extraction, lyophilization for concentration, and HPLC of the reconstituted samples using strong-cation exchange chromatography, isocratic elution, and absorbance measurement. Huperzine A was resolved with F-substituted benzene reverse phase chromatography and measurement by HPLC-MS. The linear dynamic range of sensitivity covers at least 500 to 0.5 ng of PB and 250 ng to .038 ng of huperzine A (not shown).

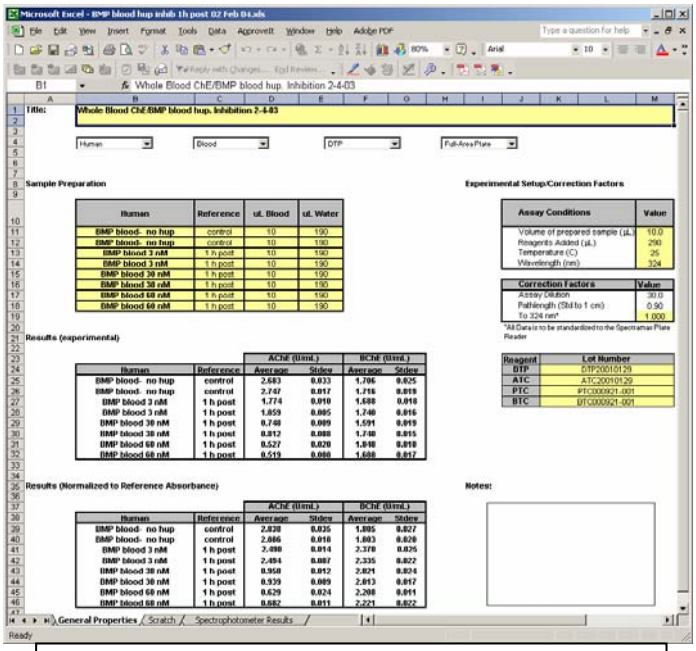

Figure 3. WRAIR Spreadsheet (see Methods, 3.2)

4. RESULTS

4.1 PB and huperzine A inhibition of ChEs in blood: Figure 4 illustrates the inhibition of whole blood AChE and BChE in the nineteen human volunteers who received PB. Each red circle represents data from a single individual. Maximal AChE inhibition is observed at 2.5 h post PB dosing, with return to pre-dosing levels achieved after 24 h. At 2.5h, nineteen PB-dosed individuals yielded a mean inhibition of AChE of 27 %, with a range of inhibition of approximately 16 to 40%. The blue squares in figure 4 represent the individuals receiving only the placebo with little change in AChE activity being observed. Serum BChE levels were less affected by PB, approximately 12% overall inhibition observed after 2.5 h (not shown). PB-induced reversible AChE inhibition is variable, and could be due to a number of factors including individual PB absorption, weight, gender, age, or other variables including food intake. Nevertheless, as long as the blood samples were kept frozen at -80°C for more than six months, the PB inhibition of AChE remained unchanged (data not shown).

Analogously, figure 5 illustrates the inhibition of whole blood AChE by huperzine A in healthy elderly human volunteers in a FDA trial to evaluate its safety for Alzheimer's disease therapy. The inhibition of RBC-AChE is seen for the twelve individuals (red circles) given the increasing doses of huperzine A (200 µg dose shown). The closed squares (in blue) represent data from the three individuals given a placebo. With a 50 µg dose, a maximal reduction in RBC-AChE activity of about 18 % is seen after 1 h with activity returned to normal about 12 h later. In contrast, the 200 µg dose (given in Week 4) produced a much larger reduction in activity (approximately 53 %) 2 h after the last dose was administered. It can also be seen that RBC-AChE activity returns to normal rather slowly, with about 25% inhibition still being observed 24 h later (figure 5). Huperzine A, even at 200 µg, had no effect on BChE levels (data not shown).

4.2 RBC-AChE protection assays to *ex vivo* GD by PB and huperzine A. PB carbamylates and huperzine A reversibly binds to AChE, and thereby protects the enzyme from reaction with OPs. The activity of the PB or huperzine A-protected but inhibited AChE will be restored once the drug-AChE complex spontaneously decarbamylates (PB) or dissociates (huperzine A), which occurs after GD is cleared from the blood. To illustrate this, the maximally PB-inhibited RBC-AChE (2.5 h post-dose, figure 4) or huperzine A-inhibited RBC-AChE (1.5 h, figure 5) were exposed to GD *ex vivo*. Next, the samples were rapidly centrifuged through a column to remove any free GD and drug, which bind to the column matrix while allowing all the ChEs to pass through the column. Under these cir-

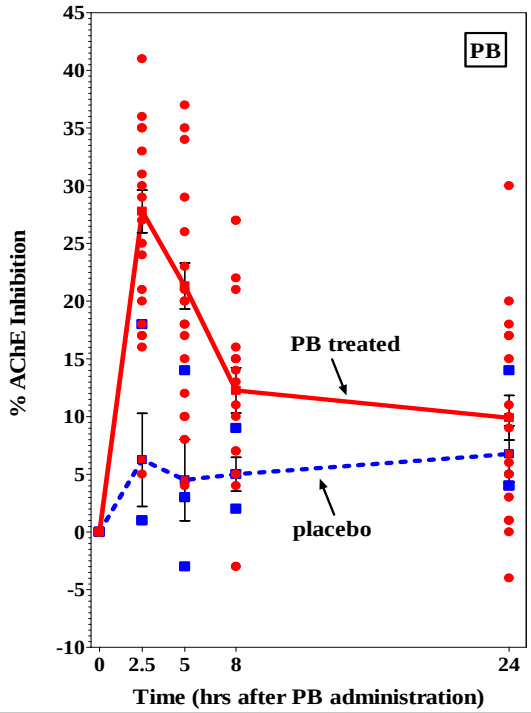

Figure 4. Time course of inhibition of RBC-AChE in humans given a single 30 mg dose of pyridostigmine bromide (PB). Nineteen volunteers received PB (red circles), with five receiving a placebo (blue squares). ChE were measured using the WRAIR WB assay.

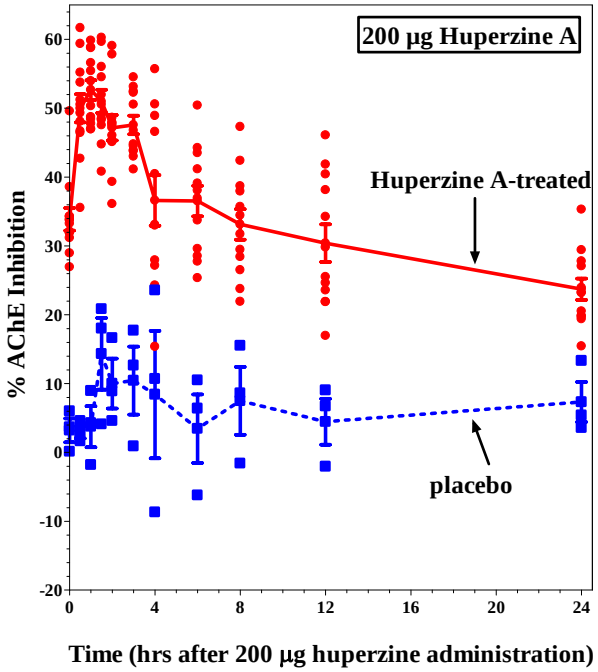

Figure 5. Time course of inhibition of RBC-AChE in humans given an increasing dose of huperzine A. Twelve volunteers received the drug (red circles), and three receiving a placebo (blue squares). ChE were measured using the WRAIR WB assay.

cumstances, any RBC-AChE not protected by PB or huperzine A would be irreversibly inhibited by GD. In contrast, the RBC-AChE protected by PB/huperzine A would spontaneously decarbamylate/dissociate over time, and this enzyme's activity would be restored. In figure 6 and figure 7, the red bars represent RBC-AChE without any PB (placebo), while the blue bars represent AChE from an individual receiving the 30 mg PB tablet or 200 µg huperzine A. In the first part, after GD treatment, no AChE activity is observed by the WRAIR assay in either the placebo or drug treated volunteers (red bars close to 0 U/mL). However, after the spin column removal of free PB and GD, and a 24 h period to allow for complete decarbamylation, the PB-inhibited AChE is restored to the level that was initially inhibited by PB (29.9% inhibition by PB before the column vs. 33.7% returned AChE activity post-column; figure 6). Also shown in figure 6 is that the recovery of AChE activity is almost complete after 3 h. These results definitively demonstrate that RBC-AChE is protected by PB from *ex vivo* GD.

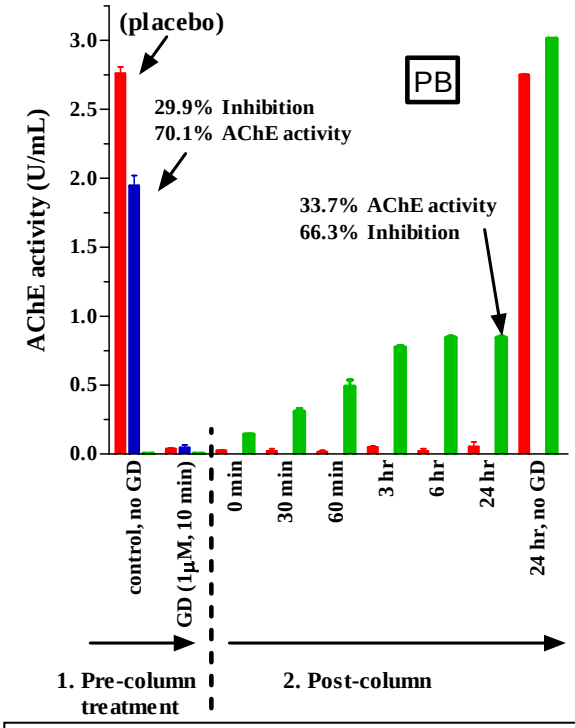

Figure 6. Effects of GD on AChE activity in whole blood from human volunteers who had taken PB (30 mg tablet). Blood was drawn 2.5 hr post-dose when RBC-AChE is maximally inhibited by PB (blue bar, left of dashed line). After GD exposure, PB and GD were removed using a C_{18} chromatography spin column (post-column treatment). The PB-protected AChE activity returned by 6 hr post-column. Green bars show the correlation between the initial % inhibition of RBC-AChE by PB (solid red line) and the subsequent return of AChE activity due to decarbamylation of the protected enzyme after removal of PB and GD (green bars).

Similar results can be observed for huperzine A protection of RBC-AChE (figure 7). In this case, huperzine A (200 µg) was a more potent inhibitor than PB, yielding 60% inhibition. However, after the column for removal of free huperzine A and GD (figure 7), and a 4 h period to allow for complete dissociation, the huperzine A-inhibited AChE was almost restored to the level that was initially inhibited by the drug (60% inhibition by the drug before the column vs. 54% returned AChE activity post-column). This suggests that huperzine A is highly effective in protecting RBC-AChE from *ex vivo* GD exposure.

4.3 Correlation of PB and huperzine A concentration in blood and RBC-AChE inhibition (figure 8): The PB/huperzine A concentrations in the same blood samples used to determine RBC-AChE inhibition were analyzed by our HPLC or HPLC/MS methods (see Methods, 3.6). A linear correlation (r^2 = 0.98) was established between the amount of PB measured in the blood in all nineteen treated subjects and the % inhibition of RBC-AChE. The amount of PB in the blood ranged between 0 and 17 ng/mL. A linear correlation (r^2 = 0.87) was also established between the amount of huperzine A in the blood and % RBC-AChE inhibition. Levels of huperzine A ranged between 0.3-3.5 ng/mL. It is clear that huperzine is

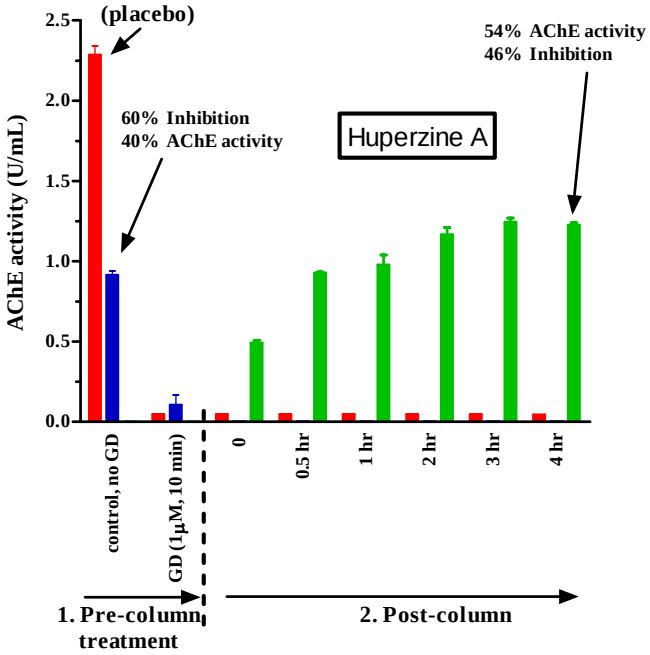

Figure 7. Same legend as figure 6, except an increasing dose of huperzine A (see Methods 3.4) was given to the human volunteers and blood was drawn 1.5 hr after the final 200 µg dose.

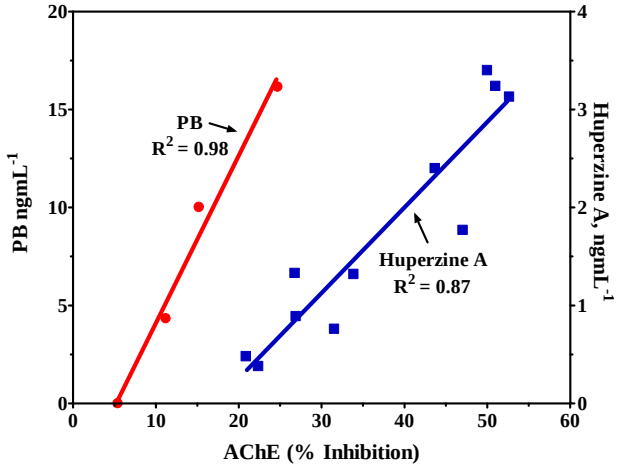

Figure 8. Correlations between % RBC-AChE inhibition (x-axis) and 1) concentration of PB in the whole blood (left axis, red circles) and 2) concentration of huperzine A in whole blood (right axis, blue squares).

a more potent RBC-AChE inhibitor than PB since smaller concentrations of the drug were required to achieve greater RBC-AChE inhibition.

5. CONCLUSIONS

The WRAIR whole blood cholinesterase assay is capable of providing fast, precise, and accurate AChE and BChE measurements, and can be directly correlated for cholinesterase activity with other more classical measurement techniques. Unlike the conventional clinical tests, the WRAIR WB procedure provides a more detailed picture of the patient's cholinesterase levels (i.e., by measuring both AChE and BChE simultaneously), produces rapid results, and is capable of high-throughput screening of whole blood by employing state-of-the-art robotics with associated precision. This method can thus rapidly establish a military or civilian personnel's exposure to insecticides, nerve agents, medicinally administered drugs (such as PB and huperzine A), narcotics, and anesthetics.

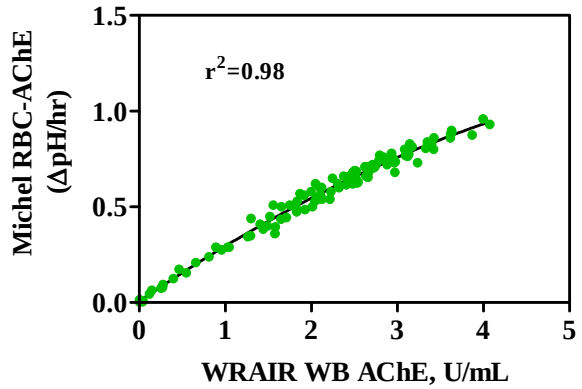

Figure 9. Correlation of the WRAIR whole blood and Michel assays.

We demonstrated (Feaster et al, 2000) co-linear relationships between cholinesterases measured by the WRAIR WB assay and four widely used alternate methods: time-modified Michel (Δ pH) method used by the U.S. Army, COBAS-FARA, Ellman and microEllman colorimetric assays, and the field Test Mate OP kit. Excellent correlations for the fitted curves to the data (r^2-values ranging from 0.94 to 0.99) were found. Figure 9 shows the correlation between the WRAIR WB and Michel. Thus, if the WRAIR WB assay is used to determine the RBC-AChE, the value can be converted to ΔpH units by applying a simple transformation defined by the line in this figure. This process would facilitate the transition from the familiar unit of Δ pH/h for the Michel method to the WRAIR WB international measurement of U/mL.

The ChE assay is in development for individual field deployment as a minimally intrusive, painless, handheld, and battery operated device that is easy to use. A novel hollow silicon needle, adapted from diabetic blood glucose monitoring, draws whole blood by capillary action into a microchip sampling chamber (Figure 10, U.S. Patent #5,801,057). In use, the micro-needle punctures the skin and draws nanoliter quantities of blood in only 10s, and then the assay is performed automatically. Coupled to an electrochemical detection system, this device will measure ChE activity in whole blood for immediate evaluation of exposure to OPs and pesticides by soldiers, first responders, or medical personnel treating patients with cholinesterase inhibitors while in the field.

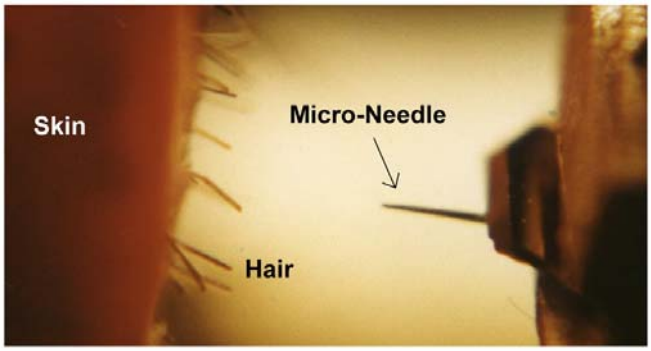

Figure 10. Silicon needle for painless field sampling of human blood ChEs (Kumetrix, Inc).

The recommended dose of PB as a pretreatment for OP exposure is 90 mg per day (3 x 30 mg at 8 h intervals; http://www.fda.gov/cder/drug/infopage/Pyridostigmine_Bromide/default.htm). After a single 30 mg dose to human volunteers, we have demonstrated that the maximal inhibition of RBC-AChE (average 27%) occurs after 2.5 h, and that this reversibly inhibited PB-AChE complex can spontaneously decarbamylate resulting in the loss of PB inhibition and the restoration of the original AChE activity. In contrast, there was no return of AChE

activity after GD exposure *ex vivo* in the human blood of volunteers receiving the placebo.

Huperzine A is a reversible and highly selective AChE inhibitor compared to BChE (Jiang, 2003), and we demonstrated that it is a more potent inhibitor of RBC-AChE than PB. Huperzine A is available as a "nutraceutical", a natural supplement reported to improve memory, and has a variety of neuroprotective effects (Gordon et al, 2001). Huperzine A is presently undergoing clinical trials as a potential treatment for Alzheimer's disease, which provided us the opportunity to measure huperzine A inhibition of RBC-AChE of healthy elderly volunteers using the WRAIR WB assay. Huperzine A exhibited no apparent side effects, even when they received 200 µg of huperzine A. This produced more than 50% inhibition of RBC-AChE (figure 5). Like PB, we demonstrated that the huperzine A-AChE complex spontaneously dissociated following *ex vivo* exposure to GD after spin column treatment, and the original protected RBC-AChE activity was restored. In contrast to PB, huperzine A passes the blood brain barrier and would protect both the CNS and peripheral AChE. We established a good correlation between the % RBC-AChE inhibition by PB and huperzine and % brain and guinea pig diaphragm muscle AChE inhibition (not shown). Therefore, the WRAIR RBC-AChE assay is a reliable surrogate for CNS and peripheral exposure to OPs. Using a sensitive HPLC method to quantify drug levels in plasma, we found excellent agreement between the levels of the two drugs and the RBC-AChE inhibition observed using the whole blood assay.

These data support the premise that pyridostigmine bromide is an effective drug for prophylaxis against the lethal effects of GD nerve agent poisoning. Given the extensive cumulative experience with the use of PB in patients with myasthenia gravis (Juhn et al, 1993) and the significantly higher doses prescribed over many years (up to 1.5 g/day), PB is a safe drug when used as a pretreatment for OP poisoning. Likewise, huperzine A is a potential drug for prophylaxis for organophosphate poisoning. It displays higher specificity for AChE than PB and has a longer biological half-life (Lallemont et al, 2002).

In conclusion, given the potential increase in urban terrorism that may include the use of chemical warfare organophosphate agents, Federal, State, and local authorities need a reliable, fast, inexpensive, and standard method for confirming such an assault in order to initiate appropriate containment, decontamination, and treatment measures. The WRAIR whole blood assay fulfills these requirements.

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Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 1996 edition. These studies were approved by the WRAIR and GU human use review committees.

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